NKG2D Ligands Expression and NKG2D-Mediated NK Activity in Sezary Patients

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Sezary syndrome (SS) is a rare lymphoma characterized by the clonal expansion in the skin and in blood of $CD4^+CD158k^+$ T cells. Natural killer (NK) activation against tumors in leukemia models is partly based on the recognition of the target through the NKG2D/NKG2D ligands interactions. We analyzed *ex vivo* SS malignant lymphocytes for the expression of the NKG2D ligands such as the major histocompatibility complex class I-related molecules (MIC) A and B and the UL16 binding proteins (ULBP). The expressions of NKG2D, the natural cytotoxicity receptors (NKp30, NKp44, and NKp46) and the activating receptor DNAM-1 were simultaneously investigated on circulating patients NK and $CD8^+$ nonmalignant lymphocytes. Interestingly, although at least one of the NKG2D ligands was expressed on the circulating malignant lymphocytes of 9 out of 10 patients, NKG2D was expressed on effector lymphocytes. We found that soluble MICA in patient's sera was increased, which may constitute a mechanism to escape the immune response. *In vitro*, SS tumor lymphocytes induced the degranulation of perforin by the NKL cell line. More importantly, NKG2D expressed on SS patients NK cells is functional and capable to induce degranulation. Altogether, these data could suggest that stimulating NK function in SS patients may be a promising strategy to reduce tumor invasion.

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INTRODUCTION

Sezary syndrome (SS) is an orphan disease corresponding to an advanced form of cutaneous T-cell lymphoma with malignant T cells in peripheral blood derived from the skin-invasive T-cell clone (Kim *et al.*, 2005; Querfeld *et al.*, 2005). SS commonly displays the expansion of a $CD45RO^+CD4^+$ clonal T-lymphocyte population expressing skin-homing receptors such as CCR4 and CCR10 as well as the cutaneous lymphocyte antigen. Moreover, we recently reported that Sezary tumor clones are characterized by the expression on the cell surface of the killer immunoglobulin-like receptor (KIR) CD158k (Poszepczynska-Guigne *et al.*, 2004), which allows for the evaluation of the tumor burden (Ortonne *et al.*, 2006; Bahler *et al.*, 2008). In addition, in some patients, expression of KIR receptors CD158a and/or CD158b on malignant $CD4^+$ T cells with an unusual activating function has also been reported (Marie-Cardine *et al.*, 2007). Immunity in SS demonstrates a severe defect in mounting a Th1 response correlating with depressed IL-12 levels. The addition of IL-12 *in vitro* to circulating lymphocytes from SS patients can reverse this Th1 defect by stimulating IFN-γ production and natural killer (NK) cell activity (Rook *et al.*, 1995, 1996). In addition, IL-21 has recently been described as a potent stimulator of NK and $CD8^+$ T-cell activity in SS peripheral blood lymphocytes without inducing a malignant T-cell proliferation (Yoon *et al.*, 2008).

Initially, NK cells were characterized by their ability to kill tumor cells and their antitumor activity is now widely accepted. Indeed, the anti-leukemia response by NK cells has strictly been demonstrated in allogeneic hematopoietic stem cell transplantation situations (Ruggeri *et al.*, 2002). NKG2D is a major activating receptor, expressed on NK and $CD8^+$ T cells, involved in tumor immunosurveillance (Coudert and Held, 2006) and whose expression is increased by IL-15 (Sutherland *et al.*, 2006). It activates effector cells in humans through the recognition of the major histocompatibility complex-class I-related molecules A and B (MICA/B) or by the UL16 binding proteins (ULBP) on target cells. NKG2D ligands (NKG2D-L) are often overexpressed on tumor cells

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Abbreviations: HD, healthy donors; K562, keratin 562; KIR, killer immunoglobulin-like receptor; MICA/B, major histocompatibility complex class I-related molecules A and B; NK, natural killer; NKG2D-L, NKG2D ligands; sMICA, soluble MICA; PBMC, peripheral blood mononuclear cells; SS, Sezary syndrome; ULBP, UL16 binding protein

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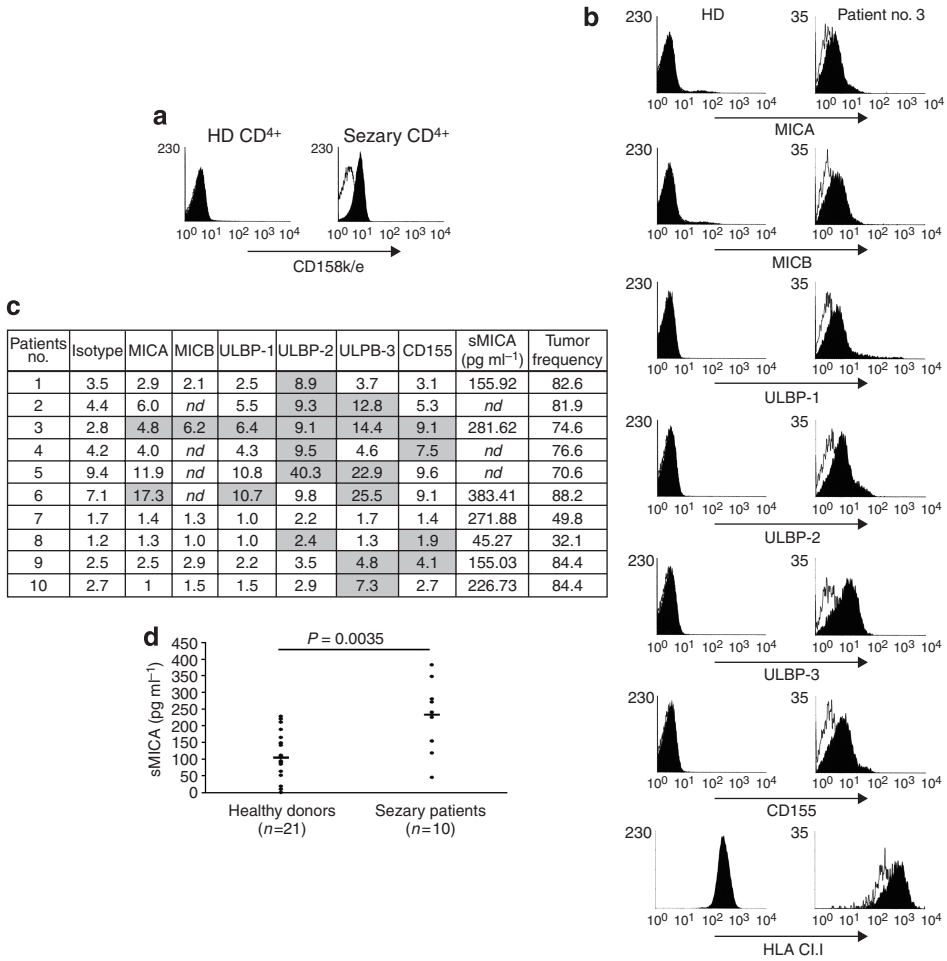
from various cytological origins, including chronic and acute leukemia (Pende *et al.*, 2002; Salih *et al.*, 2003; Diermayr *et al.*, 2007). In chronic myeloid leukemia, the NKG2D/MICA interaction may participate to the development of an antitumor response (Salih *et al.*, 2003; Boissel *et al.*, 2006). However, tumors may impair the NKG2D-mediated response by shedding MIC molecules expressed on tumor cells with production of high levels of soluble MICA and/or MICB, in addition to initiating NKG2D downregulation (Groh *et al.*, 2002; Salih *et al.*, 2003; Coudert and Held, 2006).

Previous results indicate that Sezary malignant cells are susceptible to NK lysis from activated autologous NK cells (Bouaziz *et al.*, 2005). In analyzing the expression of NKG2D-L on SS tumor cells as well as the expression and function of NKG2D on effector lymphocytes in SS patients, we determined whether the malignant cells could induce NK-mediated immune response through NKG2D/NKG2D-L interactions.

RESULTS AND DISCUSSION

Sezary tumor cells express some NKG2D-L

The staining of CD158k on the surface of tumor cells is consistent with previous data (Ortonne *et al.*, 2006; Bahler *et al.*, 2008). In addition, we show here the expression of NKG2D-L in contrast to normal healthy $CD4^+$ T cells (Figure 1). We determined, in 10 patients, expression of MICA, MICB, ULBP, and DNAM-1 ligand poliovirus receptor CD155, using flow cytometry analysis by specifically gating the $KIR3DL2/CD158k^+$ tumor cells (Figure 1b and c). Nine patients expressed at least one NKG2D-L. Sezary cells were primarily positive for ULBP-2 or ULBP-3 and 3 out of 10 tumors presented both. MICA, MICB, and ULBP-1 were expressed on some Sezary tumors as well as CD155. Only one patient expressed all of the NKG2D-L. HLA class I molecules were detected on all SS tumor cells with similar levels as opposed to peripheral blood mononuclear cells



Patients no.	Isotype	MICA	MICB	ULBP-1	ULBP-2	ULPB-3	CD155	sMICA (pg ml^{-1})	Tumor frequency
1	3.5	2.9	2.1	2.5	8.9	3.7	3.1	155.92	82.6
2	4.4	6.0	*nd*	5.5	9.3	12.8	5.3	*nd*	81.9
3	2.8	4.8	6.2	6.4	9.1	14.4	9.1	281.62	74.6
4	4.2	4.0	*nd*	4.3	9.5	4.6	7.5	*nd*	76.6
5	9.4	11.9	*nd*	10.8	40.3	22.9	9.6	*nd*	70.6
6	7.1	17.3	*nd*	10.7	9.8	25.5	9.1	383.41	88.2
7	1.7	1.4	1.3	1.0	2.2	1.7	1.4	271.88	49.8
8	1.2	1.3	1.0	1.0	2.4	1.3	1.9	45.27	32.1
9	2.5	2.5	2.9	2.2	3.5	4.8	4.1	155.03	84.4
10	2.7	1	1.5	1.5	2.9	7.3	2.7	226.73	84.4

Figure 1. Expression of NKG2D-L on SS tumor cells and sMICA in patient's sera. (**a**) *Ex vivo* CD158k/e staining of $CD4^+$ T lymphocytes in one HD and one patient (filled histogram). In the Sezary patient, CD158k/e expression was compared to $CD4^-$ T cells (empty histogram). (**b**) *Ex vivo* phenotype profile on $CD158k^+$ tumor cells from one representative patient (no. 3) as compared to $CD4^+$ T cells in one HD. Expressions were compared on $CD4^+CD158k^+$ tumor cells (filled histogram) and on the $CD158k^-$ lymphocytes (empty histogram). (**c**) NKG2D-L and CD155 expression profiles were analyzed in 10 SS patients. Numbers indicate the median of fluorescence intensity for each marker. A grey box shows the positive signal defined as superior by at least 1.5-fold to the isotype control. Concentrations of sMICA and frequency of tumor cells in lymphocytes have been indicated when available. (**d**) Concentrations of sMICA in sera from patients ($n=10$) and HD ($n=21$) were significantly increased in SS patients; nd, not determined.

Table 1. Frequency of marker$^+$ cells in Sezary syndrome patients (n=6) and healthy donors (n=21)

	Patients no.							HD
	1	2(%)	3	4(%)	5(%)	6	Median(%)	Median(%)
$CD3^-CD56^+$ NK cells								
NK cells/lymphocytes	3.9%	2.0	6.3%	0.1	0.8	0.4%	2.3	3.8
$NKG2D^+$/NK cells	49.8%	25.6	90.2%	77.9	65.3	22.6%	57.6	53.7
$NKp30^+$/NK cells	ND	1.2	ND	39.2	43.3	27.4%	33.3	31.5
$NKp44^+$/NK cells	ND	0.6	ND	17.6	15.3	12.2%	13.7	3.3
$NKp46^+$/NK cells	ND	2.2	ND	54.4	66.0	26.1%	40.2	51.3
DNAM-1^+/NK cells	ND	59.1	ND	14.3	69.5	63.4%	61.2	53.6
$CD8^+$ T cells								
$CD8^+$/lymphocytes	5.9%	2.5	7.9%	0.9	0.1	1.2%	3.1	17.4
$NKG2D^+/CD8^+$ T cells	58.4%	71.7	90.2%	87.6	57.0	79.2%	75.5	62.0
DNAM-$1^+/CD8^+$ T cells	ND	17.2	ND	42.2	50.0	ND	42.2	36.8

ND: not determined.

(PBMC) from healthy donors (data not shown). Production of soluble forms of MICA (sMICA) by tumors may constitute a mechanism to escape the immune system (Groh *et al.*, 2002; Salih *et al.*, 2003), for this reason we quantified the levels of sMICA in patients' sera in a comparison to healthy donors (HDs; Figure 1c and d). Sezary patient's sera were significantly more enriched in sMICA as compared to HD (medians: 222.7 and 108.67 pg/ml, respectively, $P=0.0035$). Although increased sMICA levels did not directly correlate with MICA expression on the tumor, we observed that patients with $MICA^+$ tumors ($n=2$) had the highest sMICA concentrations (383.41 and 281.62 pg ml^{-1}; Figure 1c).

NKG2D expression on NK and T $CD8^+$ lymphocytes of Sezary patients

NK and T lymphocytes express a panel of activating receptors such as NKG2D, DNAM-1, and the natural cytotoxicity receptors (NKp30, NKp44, and NKp46), the latter commonly being absent on T lymphocytes. Downregulation of such receptors in SS patients might impair the antitumor response. NK lymphocytes defined as $CD3^-CD56^+$ lymphocytes and T $CD8^+$ (which altogether may represent less than 1% of PBMC besides the $CD4^+CD158k^+$ malignant cells; Table 1) were phenotyped by flow cytometry in six SS blood samples. All receptors were expressed on NK cells, whereas only NKG2D and DNAM-1 were expressed by $CD8^+$ T cells (Table 1; Figure 2a). Levels of expression showed a broad variability with, in some cases, a severe downregulation of NKG2D (patients 2 and 6), DNAM-1 (patient 4), or NKp30 and NKp46 (patient 2). Although median levels were not significantly different between HD and SS patients, these observations might be of importance in the context of an individual antitumor response. In contrast, NKp44 expression seemed to be increased in three out of four patients tested, probably indicating the activated status of NK cells (Vitale *et al.*, 1998). NKG2D expression on $CD8^+$ T cells was less variable than on NK lymphocytes. Interestingly, patients with reduced NKG2D expression on NK cells kept a normal expression on $CD8^+$ T lymphocytes (Figure 2c), highlighting the differences in the regulation of NKG2D expression in both lymphocyte populations. There was no correlation between the expression of NKG2D and the frequency of tumor cells in the periphery or the levels of sMICA. However, sMICA is not the only mechanism for the tumor to escape the NK immune response. Indeed, the high expression of NKG2D-L on tissues may induce the downregulation of NKG2D (Oppenheim *et al.*, 2005) and therefore may constitute for tumors with high invasive rate an efficient mechanism to repress antitumor activity. Tissue growth factor-β is also known to downregulate NKG2D expression on NK cells in concordance with a direct impact on NK activity against tumor cells in human (Lee *et al.*, 2004). However, quantification of tissue growth factor-β in Sezary patients' sera (median: 36.66 pg ml^{-1} (4.55–43.95 pg ml^{-1})) was similar to HD (29.34 pg ml^{-1} (23.89–39.19 pg ml^{-1})).

Sezary tumor cells activate NK cell degranulation

The degranulation of perforin and granzymes by NK cells can be assessed by detecting the cell-surface expression of the lysosomal-associated membrane protein CD107a, in a flow cytometry-based assay (Bryceson *et al.*, 2005). The detection of CD107a correlates with the lysis of the target by effector cells (Bryceson *et al.*, 2005). To analyze the sensitivity of *ex vivo* freshly isolated Sezary lymphocytes through the recognition of NKG2D-L, we used the NK cell line NKL in CD107a mobilization assays. NKL was chosen because of its high expression of NKG2D with a minimal expression of natural cytotoxicity receptors (Boissel *et al.*, 2006; data not shown). In presence of the Sezary tumor cells ($n=5$), about 8% of NKL cells mobilized of CD107a on their surface, which is equivalent to the degranulation against the class I negative NK target keratin 562 (K562; Figure 3a). This

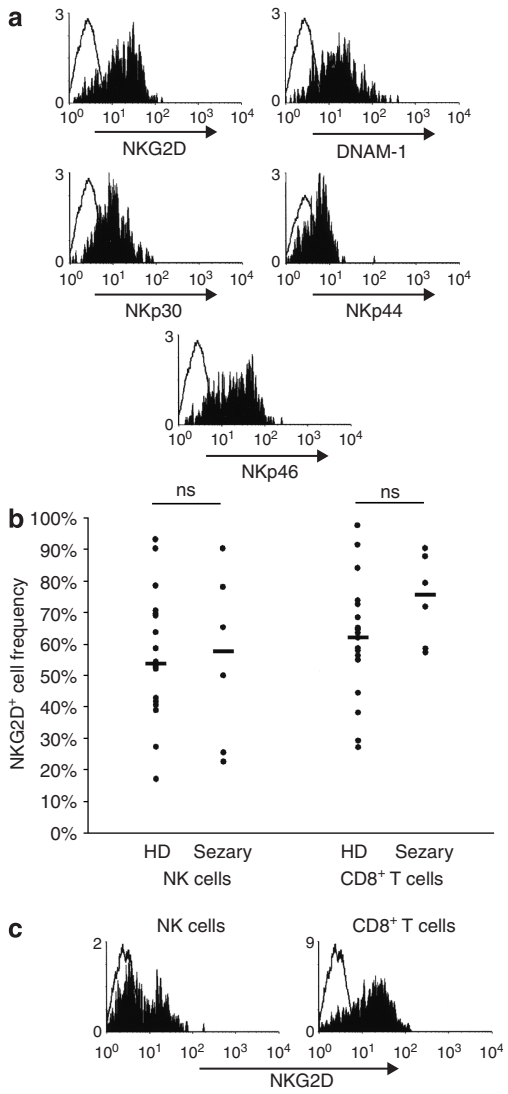


Figure 2. Expression of activating NK receptors on effector cells in SS patients and HD. (**a**) *Ex vivo* expression profile of activating NK receptors on $CD56^+CD3^-$ NK cells in a representative SS patient (no. 5, filled curve) was compared to the isotype control (empty curve). (**b**) Frequency of NKG2D-positive NK and $CD8^+$ T cells in SS patients ($n=6$) as compared to HD ($n=21$). Medians for each group are shown as a bar. (**c**) NKG2D expression (filled curve) on NK and $CD8^+$ T cells in a representative SS patient (no. 2) compared to the isotype control (empty curve).

demonstrated the capacity of SS tumor cells to trigger degranulation in an activated NK cell line.

SS patients' NK cells can be activated through NKG2D

To investigate the activation of NK cells after NKG2D engagement in SS patients, we performed a CD107a degranulation assay coupled with a NKG2D staining (Figure 3b–d). NK cells of SS patients were functional in the degranulation assay against K562 (Figure 3b and c). Moreover, degranulation was significantly increased against C1R-MICA as compared to C1R. In parallel, NKG2D expression was reduced by the interaction of NK cells with MICA-expressing targets such as K562 or C1R-MICA and not with C1R (Figure 3d). Altogether, these results illustrated the ability of NKG2D to specifically trigger SS patient's NK cells degranulation.

CONCLUDING REMARKS

This study highlights the potential role of NKG2D/NKG2D-L interactions in NK immune responses in SS patients. Our results demonstrated NKG2D-L surface expression on SS tumors and the ability of SS patient's NK cells to engage NKG2D and to be functional against sensitive targets. However, the obvious inefficiency *in vivo* of NK cells in patients underlines the importance of mechanisms used by the tumor to escape the NK-mediated antitumor response, such as sMICA production, NKG2D downregulation, or inhibition through the recognition of self HLA class I molecules by regulatory NK receptors such as the KIR or CD9/NKG2A. This provides a rationale to reduce KIR activity by using specific mAb (Koh *et al.*, 2001) or to increase NKG2D expression and function of autologous or allogeneic NK cells, for instance via IL-15 which enhances *in vitro* NK activation towards SS malignant targets (Wysocka *et al.*, 2004). These data also indicate that attempts to induce expression of MICA/B by chromatin remodeling agents, such as histone deacetylase inhibitors, as proposed in leukemia (Diermayr *et al.*, 2007; Kato *et al.*, 2007), may be helpful to SS patients.

MATERIALS AND METHODS

Patients and blood samples

Blood samples from 17 SS patients were collected in the Department of Dermatology, Henri Mondor-Albert Chenevier Hospital, Créteil, France. Patients have not been previously treated with chemotherapy. All patients presented in the blood dominant T-cell clones by TCR-γ chain analysis with the use of PCR-DGGE and have been previously described (Ortonne *et al.*, 2006; Marie-Cardine *et al.*, 2007). Seven samples were analyzed for phenotypes and sMICA, three patients for phenotypes only and three others to quantify sMICA. Four patients were added for the functional experiments. HD's blood samples were obtained at the Saint-Louis Hospital Transfusion Center, Paris, France. All patients and healthy controls gave their informed consent to this study, which was approved by the Institutional Review Board of the Hospital Henri Mondor, Créteil, France. This work followed the Declaration of Helsinki Principles.

PBMC were isolated from freshly collected blood samples by density gradient centrifugation using lymphocyte separation medium (Eurobio, Les Ulis, France) and stored in liquid nitrogen. Sera were collected after coagulation and centrifugation of blood samples.

Flow cytometry analysis

Immunophenotyping analyses were performed on frozen samples of PBMC on a BD Biosciences LSR or a FACSCanto II flow cytometer (Becton Dickinson, Le Pont de Claix, France).

Lymphocyte immunophenotyping was performed in four- or six-color analysis with the following mAb: CD8-FITC produced by R&D Systems (Lille, France); NKG2D-PE, NKp30-PE, NKp44-PE, and NKp46-PE from Beckman Coulter (Villepinte, France); DNAM-1-PE, CD3-PerCP, CD8-PerCP, CD3-AmCyan, CD4-allophycocyanin, CD16-PacificBlue, and CD56-allophycocyanin commercialized by

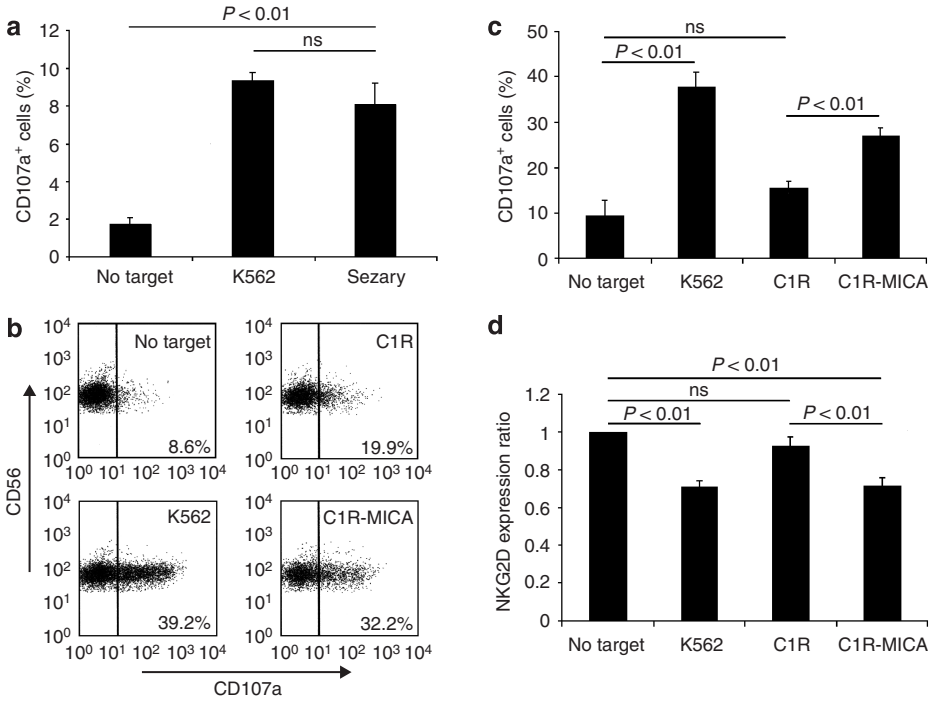


Figure 3. NK function in SS patients. (**a**) Frequency of CD107a$^+$ NKL cells after coculture with SS tumor cells ($n = 5$), K562 or without target. (**b**) Detection of CD107a$^+$CD3$^-$CD56$^+$ NK cells from one representative SS patient after stimulation by K562, C1R, or MICA-transfected C1R (C1R-MICA) cell lines. (**c**) Means of frequency of CD107a$^+$CD3$^-$CD56$^+$ NK cells of four SS patients after stimulation by the three targets. (**d**) Ratios of NKG2D mean of fluorescence intensity of NK cells incubated with and without target ($n = 4$). Histograms show means ± s.e.m; ns, not significant.

Becton Dickinson. To study tumor cells, indirect stainings were combined. Binding of mAbs specific for MICA, MICB, ULBP-1, ULBP-2, and ULBP-3 (R&D Systems), HLA class I molecules and CD155 (Beckman Coulter) was revealed with a PE-labelled goat anti-mouse IgG-specific mAb. CD158k was detected on tumor cells with the IgM mAb Q66 (Ortonne *et al.*, 2006) together with a FITC-labelled IgM-specific secondary mAb (Jackson ImmunoResearch, Soham, UK). CD158k/e has been detected in patients or HD CD4$^+$ T cells with the IgG mAb AZ158 (Bagot *et al.*, 2001). Both CD158k-specific mAb were provided by Professor A. Moretta. Proper IgG and IgM isotype controls were used and the expression of NKG2D-L on SS tumors was taken as positive when the median of fluorescent intensity was increased 1.5-fold compared to the isotype control.

ELISA

sMICA was quantified in −80 °C stored patients and HD sera with ELISA kits provided by Bamomab (Munich, Germany) and according to manufacturer's recommendations.

Degranulation assay

To test the ability of patients' tumor cells to induce NKL degranulation, target cells K562 and patients' PBMC were incubated with the NKL cell line deprived of IL-2 for 2 days before the experiment. Evaluation of SS patient's NK cells activity was performed with unsorted SS peripheral blood lymphocytes thawed the day before the experiment. Cocultures were performed overnight at an effector/target ratio of 1/1 at 37 °C, in culture medium in the presence of CD107a-PE (Becton Dickinson) for the term of the assay. Thereafter, cells were stained with NKG2D-allophycocyanin, CD3-PerCP (Beckman Coulter), CD56-allophycocyanin or CD56-Alexa Fluor 488 (Becton Dickinson) antibodies for 20 minutes on ice. Cells were then fixed in phosphate-buffered saline-2% paraformaldehyde and analyzed by flow cytometry. CD107a and NKG2D surface expressions on effector cells incubated without target were evaluated as the negative controls of the experiment.

Statistics

Frequencies of cell subsets, sMICA concentrations as well as results of functional assays in HD and SS patients have been compared using the Mann–Whitney test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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